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EFFECTS OF DIETARY ENERGY ON TRANSCRIPTIONAL ADAPTATIONS AND INSULIN RESISTANCE IN DAIRY COWS AND MARES

DOCTORAL THESIS

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II **Selim S., T. Kokkonen, J. Taponen, A. Vanhatalo, and K. Elo**. 2015. Effect of prepartal ad libitum feeding of grass silage on transcriptional adaptations of the liver and subcutaneous adipose tissue in dairy cows during the periparturient period. J. Dairy Sci. 98:5515–5528, In press.

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ABSTRACT

The objective of the research described in this thesis was to increase the understanding of the transcriptional adaptations of genes encoding proteins, which have key roles in lipid and glucose metabolism, insulin signalling and inflammation, in situations of either overfeeding energy or controlled energy during important periods in the life of dairy cows and mares. Emphasis was placed on the potential to decrease metabolic disorders and to improve animal nutritional management and health.

Experiments documented in publications I and II involved two nutritional studies to evaluate the effects of high energy feeding on the expression of genes that play important roles in major metabolic pathways in the liver and adipose tissue of dairy cows during the periparturient period. Sixteen Finnish Ayrshire dairy cows were divided into two treatment groups in the experiments I and II. In the experiment I, dairy cows were fed either a controlled energy diet (99 MJ/d metabolizable energy (ME)) during the last six weeks of the dry period or high energy diet (141 MJ/d ME) for the first three weeks and then gradually decreasing energy allowance during three weeks to 99 MJ/d ME by parturition. In the experiment II, dairy cows were fed *ad libitum* either grass silage (144 MJ/d ME) or a mixture of grass silage, wheat straw and rapeseed meal (TMR, 55%: 40%: 5%, 109 MJ/d ME) during the dry period. Liver and adipose tissue biopsies were collected at -10, 1 and 9 d and at -14, 1 and 7 d relative to actual calving in the experiments I and II, respectively. Blood samples were taken at -10 (I)/-14 (II), 1 and 7 d relative to actual parturition. In the experiment III, the impact of grazing either on cultivated high-yielding pasture (CG) or semi-natural grassland pasture (NG) on fat deposition, insulin resistance status and adipose tissue gene expression of Finnhorse mares was studied. Body measurements, intravenous glucose tolerance tests (IVGTT), and sampling for the determination of neck and tailhead adipose tissue gene expressions were conducted in May and September.

In the experiments I and II, overfed cows had greater total dry matter and ME intakes and ME balance before parturition than control cows, but no differences were observed after calving. There was no difference in body weight and body condition score between overfed and control cows in the experiment I. Increases in body weight and body condition score were greater in the overfed cows of the experiment II during the dry period. In the experiment I, there was greater plasma insulin and lower glucagon/insulin

ratio in the overfed cows than in the control cows, while in the experiment II, there were no differences in blood parameters between overfed and TMR group during the transition period. Down-regulation of key genes linked to hepatic gluconeogenesis and fatty acid β-oxidation in the overfed group of cows in the experiment I was found, suggesting impaired liver function compared to a controlled energy diet. In the experiment II, *ad libitum* feeding of grass silage throughout the dry period may have attenuated the increase of hepatic gluconeogenic capacity from propionate compared to a controlled TMR diet. However, there was no difference in the expression of genes related to hepatic glucose release during the transition period (II). In adipose tissue, there was some evidence that the level of energy overfeeding may have exacerbated the inflammatory status postpartum and temporarily decreased lipogenesis very near parturition relative to control energy diet (I). In the experiment II, prepartal *ad libitum* feeding of grass silage decreased lipogenesis and insulin sensitivity early postpartum compared to the TMR control group.

In the experiment III, CG mares had higher median body condition score (using the Henneke 1 to 9 scoring system) and body weight, and larger waist circumference than NG mares at the end of grazing. In September, greater basal and peak insulin concentrations, and faster glucose clearance rate during IVGTT were observed in CG mares than in NG mares. In addition, a greater decrease in plasma non-esterified fatty acids during IVGTT was noticed in CG mares. There were no differences in the expression of genes related to insulin resistance, inflammation and lipogenesis between the two groups. Significant temporal differences in the expression profiles of genes related to insulin resistance and lipogenesis were observed during the grazing season. Grazing on CG had moderate effects on responses during IVGTT, but did not exacerbate insulin resistance.

In conclusion, overfeeding energy with concomitant weight gain moderately altered the expression of genes related to insulin resistance, inflammation and lipogenesis in adipose tissue of dairy cows. Prepartal overfeeding energy affected the expression of genes related to hepatic gluconeogenesis and fatty acid oxidation in dairy cows, but the extent of these effects differed depending on the dietary composition during the close-up period (e.g. feeding of cereal grain). In mares, the diets with variable energy content did not affect the expression of insulin resistance- or inflammation-related genes, although mares were different in their body condition scores.

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ABBREVIATIONS

ACTH	adrenocorticotropic hormone
ADIPOQ	adiponectin
ADIPOR1	adiponectin receptor 1
ADIPOR2	adiponectin receptor 2
AT	adipose tissue
BCS	body condition score
BHBA	beta-hydroxybutyrate
BW	body weight
CG	cultivated high-yielding pasture
CPT1A	mitochondrial carnitine palmitoyltransferase 1A
DGAT1	diacylglycerol O-acyltransferase 1
DM	dry matter
DMI	dry matter intake
G6PC	glucose-6-phosphatase catalytic subunit
HSL/LIPE	hormone sensitive lipase
IGF-1	insulin like growth factor 1
IL6	interleukin 6
INSR	insulin receptor
IR	insulin resistance
IRS1	insulin receptor substrate 1 transcript variant 1
IVGTT	intravenous glucose tolerance test
LEP	leptin
LPL	lipoprotein lipase
LPS	lipopolysaccharide
MCP-1	monocyte chemoattractant protein-1
ME	metabolizable energy
NEB	negative energy balance
NEFA	non-esterified fatty acids
NFKB1	nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
NG	semi-natural grassland
NSC	non-structural carbohydrates
PC	pyruvate carboxylase
PCK1	cytosolic phosphoenolpyruvate carboxykinase 1
PCR	polymerase chain reaction
PPARA	peroxisome proliferator-activated receptor alpha
PPARG	peroxisome proliferator-activated receptor gamma
RBP4	retinol binding protein 4
RETN	resistin
SAT	subcutaneous adipose tissue
SCD	stearoyl Co-A desaturase
SLC22A5	solute carrier family 22 (organic cation/carnitine transporter), member 5
SLC2A2	solute carrier family 2 (facilitated glucose transporter), member 2
TAG	triacylglycerol
TMR	total mixed ration
TNFα	tumor necrosis factor-alpha
VLDL	very low density lipoprotein
α-MSH	alpha-melanocyte stimulating hormone

1. INTRODUCTION

For wild animals, it is essential to maintain a certain amount of body fat stores as a source of energy, as it helps the animals to cope with food scarceness and changes in weather conditions. However, in domestic animal species, which are kept under modern management conditions, accumulation of fat may predispose animals to obesity-associated metabolic diseases. Obesity has been shown to be associated with the inflammatory response and insulin resistance (IR) in humans (de Luca and Olefsky, 2008), pet animals and horses (Radin et al., 2009) and cows (Holtenius et al., 2003; Khan et al., 2013). The mechanisms that link obesity, IR and inflammation are partly common in humans and animals and they include especially pathways related to free fatty acids, cytokines and adipokines (Drackley et al., 2005; de Luca and Olefsky, 2006; Guilherme et al., 2008; Frank et al., 2010b; Loor, 2010; Figure 1). However, a majority of our understanding of the relationships between molecular mechanisms is based on human and laboratory animal studies. Therefore, it is important to further elucidate molecular mechanisms in domestic animal species.

In obesity, adipose tissue (AT) is under a state of metabolic stress, resulting in the activation of the inflammatory response (de Luca and Olefsky, 2006; Figure 1). Adipokines such as resistin, leptin, retinol binding protein 4 and adiponectin, which are secreted by AT, can influence inflammation and insulin sensitivity (Jung and Choi, 2014; Figure 1). As a part of the inflammatory process, locally secreted chemokines trigger additional pro-inflammatory macrophages into AT (Tateya et al., 2010). These pro-inflammatory macrophages then release cytokines that further exacerbate inflammation and IR (Jung and Choi, 2014; Figure 1). High level of pro-inflammatory cytokine tumor necrosis factor-alpha (TNFα) results in decreased fatty acid esterification and enhanced lipolysis in AT (Guilherme et al., 2008). Mobilization of fat increases the transport of non-esterified fatty acids (NEFA) to the liver, which in turn disrupts normal metabolic and secretory function and inhibits insulin signalling in the liver, thus triggering IR (de Luca and Olefsky, 2006; Guilherme et al., 2008; Figure 1).

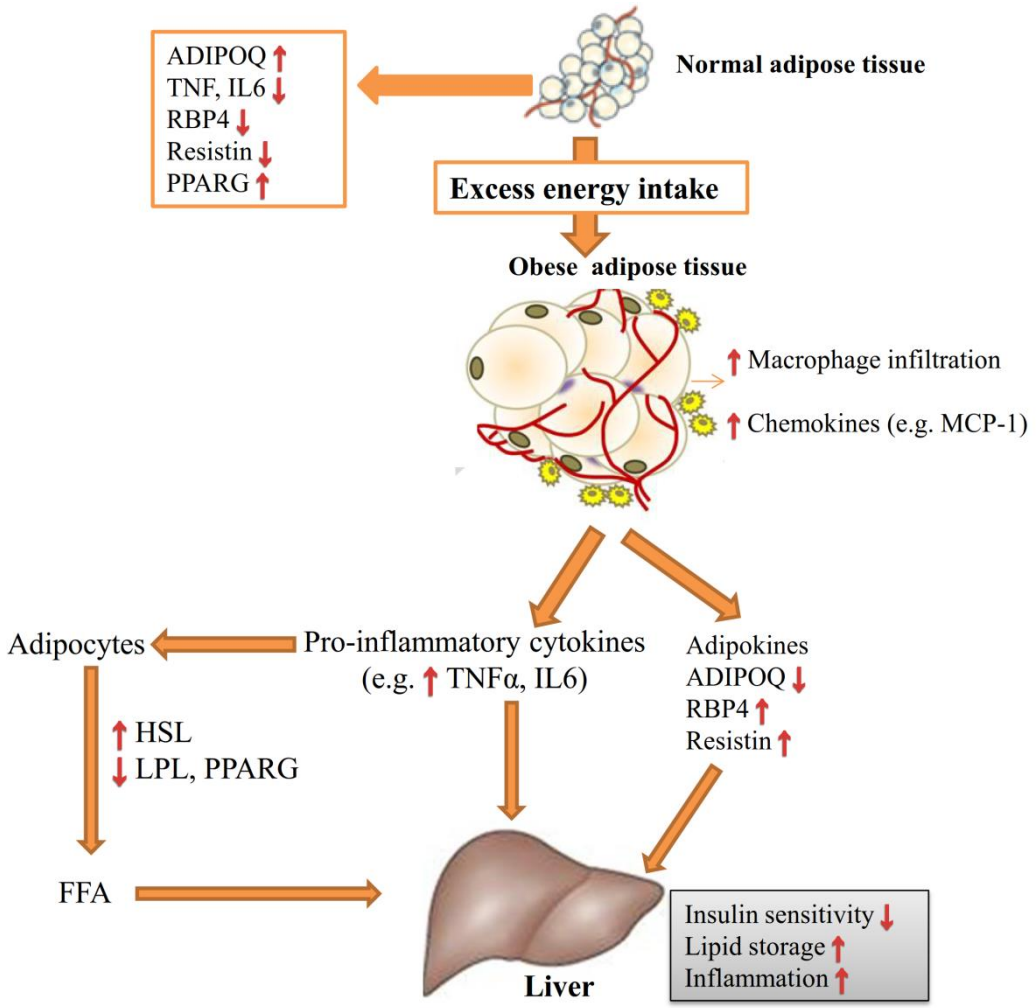

Figure 1. Schematic representation of effects of overfeeding energy on adipose tissue and the liver. ADIPOQ, adiponectin; FFA, free fatty acids; HSL, hormone sensitive lipase; IL6, interleukin 6; LPL, lipoprotein lipase; MCP-1, monocyte chemoattractant protein-1; PPARG, peroxisome proliferator-activated receptor gamma; RBP4, retinol binding protein 4; TNFα, tumor necrosis factor-alpha. Modified from de Luca and Olefsky (2006).

Transition period is the most critical period in the management of dairy cows, generally defined as 3 weeks before calving to 3 weeks after parturition (Drackley, 1999). The transition phase is characterized by a complex series of coordinated metabolic and endocrine adjustments in dairy cows (Grummer, 1995; Drackley, 1999). Failure to adequately meet the homeorhetic control of metabolism can result in a variety of metabolic and production-related diseases (Grummer, 1995; Vernon, 2005). These conditions arise from an increase in lipid mobilization, with consequent elevated plasma NEFA, and increased rate of fatty acid uptake by the liver (Grummer, 1995). In the liver, NEFA can be completely oxidized to CO_2 or incompletely to ketone bodies, or esterified

to triacylglycerol (TAG) for storage in the liver, or exported as TAG within very low density lipoprotein (VLDL) to extra-hepatic tissues such as the mammary gland (Drackley, 1999). However, if the rate of NEFA esterification in the liver for TAG synthesis exceeds the rate of TAG disappearance, accumulation of TAG and cholesterol esters in the liver take place, and may affect liver function and productivity of the cows (Grummer, 1993). It is well known that the health of transition cow is closely linked to the hepatic capacity to synchronize these processes and to cope with the metabolic changes that go together with the transition from pregnancy to lactation.

Several nutritional strategies have been developed in the dairy industry to adjust AT metabolism during the transition period with the objectives of maximizing milk production and minimizing health problems. One of the most important factors that regulates AT metabolism and liver gluconeogenic capacity of dairy cows during the transition phase is the level of energy intake prepartum. It has been suggested that maximizing energy density of the diet during the last few weeks of pregnancy would be advantageous for optimal health and production (Bertics et al., 1992; Grummer, 1995). However, the potential negative effects of overfeeding energy during the dry period on cow health and liver function have been reported (Dann et al., 2006; Douglas et al., 2006; Loor et al., 2006; Graugnard et al., 2013; Khan et al., 2014). Controlled energy feeding during the dry period has been shown to improve dry matter intake (DMI) and body condition, and minimize excessive lipolysis after parturition (Kokkonen et al., 2005; Douglas et al., 2006; Janovick and Drackley, 2010).

Horses are selective grazers, and pasture is considered to be an important feed source. Grazing is known to have benefits and is therefore recommended always when possible. Foraging is essential for normal function of the equine gastrointestinal tract (Pagan, 2009). Well-managed pastureland is an economical source of high-quality feed for horses. In the southern part of Finland horses can graze about 100-120 days (Miraglia et al., 2006), and well-managed pastures can cover about 25% of horse’s protein and energy demands per year (Särkijärvi et al., 2010). In Finland, semi-natural grassland pasture that has grown during summer (June-September) can meet horse's nutritional needs for maintenance when the grazing intensity is optimized. Some grass species such as timothy (*Phleum pratense L.*) and meadow fescue (*Festuca pratensis L.)* have been reported to

have high contents of water soluble carbohydrates and may predispose horses to metabolic disorders (Särkijärvi et al., 2010).

Equine metabolic syndrome is a complex disorder that is an endocrinopathic disease characterized by abnormal regional adiposity/obesity, IR/hyperinsulinemia, altered reproductive cycling and laminitis (Frank et al., 2010b). Laminitis is most likely associated with endocrine dysfunction, and metabolic factors such as obesity and IR are considered major predisposing factors for this health problem (Vick et al., 2007; Bailey et al., 2007; Karikoski et al., 2011). Insulin resistance appears to increase in horses fed a diet rich in non-structural carbohydrates (NSC) compared to those fed a diet high in fat and fiber (Hoffman et al., 2003). Higher blood insulin concentration was observed in obese, insulin resistant horses in comparison to lean horses (Frank et al., 2006), and this elevation of blood insulin may compensate for lower insulin sensitivity in these horses. The occurrence of IR and laminitis seems to follow a seasonal pattern, being particularly high during spring and summer (Treiber et al., 2006). These seasons are characterized by rapid grass growth and the accumulation of NSC in pasture forage is high. Another important aspect in this context is that certain breeds are more frequently affected than others (Geor, 2009); probably indicating that phenotypic or genetic factors confer susceptibility to IR and laminitis.

The study of metabolic activity of an organ in animals requires that major metabolic pathways such as lipogenesis, lipolysis, inflammation and insulin signalling in AT, and gluconeogenesis, fatty acid oxidation, insulin signalling and glucose transporters in the liver can be analysed. A variety of proteins in these metabolic pathways are under the transcriptional control, partly through the effects of the nuclear receptors such as peroxisome proliferator-activated receptor alpha and gamma (PPARA and PPARG). Quantitative Real–Time PCR is one of the main tools to study the mRNA expression. Therefore, utilization of tissue biopsies of AT or the liver with this tool may help to understand tissue metabolic adaptations to various physiological, seasonal and nutritional states.

The underlying molecular mechanisms regulating metabolic adjustments in the liver and AT of dairy cows during the transition period in the context of overfeeding energy are not fully elucidated. In addition, few studies have examined the effect of pasture feeding on the regulation of adipokines in the horse, and there is a lack of knowledge regarding the

molecular mechanistic link between increased body fatness during summer grazing and gene expression of cytokines in AT. Accumulating evidence from human and laboratory animal studies indicates that the development of IR in the context of obesity is assumed to occur in response to a state of low-grade inflammation that negatively affects insulin signalling pathways in insulin-responsive tissues, including AT and the liver. Thus, increased knowledge about the relationship between nutritional strategies and the mechanisms involved in the development of IR in dairy cows and mares can lead to significant improvements in animal management and health.

2. OBJECTIVES OF THE STUDY

The ultimate goal of the research reported in publications I-III was to increase the understanding of the transcriptional adaptations of genes encoding proteins that play important roles in fatness related metabolic dysfunctions, particularly IR, in situations of overfeeding energy during either the dry period in dairy cows or the grazing season in mares. Furthermore, the objective was also to understand the relationship of body condition to IR and SAT gene expression. For the experiments I and II, the objectives were to evaluate the effects of either overfeeding during the first three weeks of the dry period combined with gradually decreasing energy allowance three weeks before parturition or overfeeding throughout the dry period on the liver and SAT gene expression during the transition period. For the experiment III, the aim was to study the effect of grazing either on cultivated high-yielding pasture or semi-natural grassland pasture on fat deposition, insulin resistance status and SAT gene expression.

The main hypotheses tested in this research were:

- In the experiment I, overfeeding combined with decreasing energy allowance before parturition exacerbates lipid mobilization and reduces SAT insulin sensitivity in dairy cows during the periparturient period as characterized by changes in the mRNA expression of the key candidate genes in SAT. Furthermore, it was hypothesized that hepatic gene expression related to glucose metabolism and fatty acid oxidation is reduced during the transition period as a consequence of overfeeding.
- In the experiment II, *ad libitum* feeding of grass silage during the dry period enhances lipogenesis prepartum and induces more pronounced SAT mobilization postpartum as characterized by changes in SAT gene expression. In addition, overfeeding of energy alters the transcriptional activity of hepatic genes related to glucose metabolism and fatty acid β-oxidation during the transition period.
- In the experiment III, grazing on cultivated high-yielding pasture is accompanied by increased fat deposition, greater basal insulin concentration and alterations in glucose, insulin and non-esterified fatty acids responses to glucose challenge, and variation in the expression of genes potentially associated with IR in SAT.

3. MATERIALS AND METHODS

3.1 Experimental animals and treatments

The studies documented in publications I-III were conducted as three separate experiments. the experiments I and II were performed with multiparous Finnish Ayrshire dairy cows at the Viikki experimental barn during 2009-2011, and the experiment III was performed with Finnhorse mares at MTT Agrifood Research Finland in Ypäjä during 2011 (current: Natural Resources Institute Finland). An average 305 d milk yield from the previous lactation of multiparous cows was 10503 kg. Experiments were conducted in a randomized complete block design. The experimental part of the work is described here as a general outline (Table 1). A detailed descriptions of the experiments can be found in the original publications (I-III).

The experiment I evaluated the effects of overfeeding energy during the first three weeks of the dry period combined with gradually decreasing energy allowance by parturition on the liver and SAT gene expressions of dairy cows during the periparturient period. The experiment II examined the effects of *ad libitum* feeding of grass silage throughout the dry period on the liver and SAT gene expression of dairy cows during the transition period. A schematic presentation of the measurements made in the experiments I and II is given in Figure 2. Sixteen cows were divided into two treatment groups in the experiments I and II. The experiments started 44 ± 5 d (I) and 58.2 ± 4.89 d (II) (mean ± SD) prior to the actual calving date. After parturition, all cows were offered the same lactation diet in both experiments.

In the experiment I, dairy cows were fed either a controlled energy diet (99 MJ/d metabolizable energy (ME)) during the last six weeks of the dry period or a high energy diet, *ad libitum* grass silage (141 MJ/d ME) for the first 3 weeks and then gradually decreasing energy allowance to 99 MJ/d ME by calving. Both groups were fed grass silage during the early dry period, and grass silage supplemented with commercial concentrate (30% of energy) during the last 3 weeks of pregnancy (I). In the experiment II, dairy cows were fed *ad libitum* either grass silage (144 MJ/d ME) or total mixed ration (TMR, 109 MJ/d ME) during the dry period. During close-up period commercial concentrate mixture was added to both groups starting from 1 kg/d at d -10 to d -6 before the expected calving date and 2 kg/d until parturition.

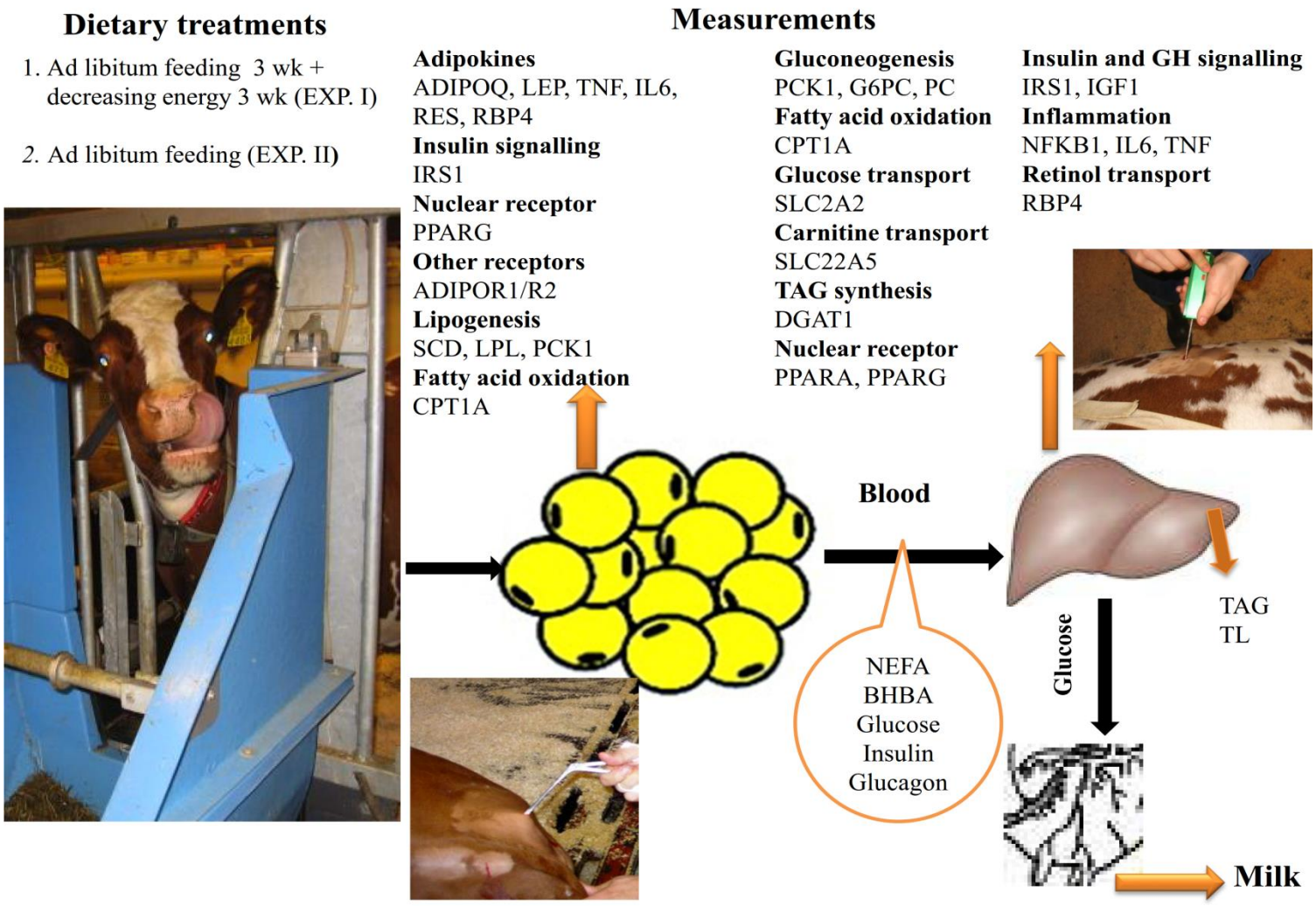

Figure 2. A schematic presentation of the measurements made in the experiments I and II. Photos were taken by Tuomo Kokkonen and Siru Salin.

The experiment III was conducted to evaluate the effect of grazing on fat deposition, glucose tolerance and SAT gene expressions, as well as to analyze associations between pasture-associated changes in body condition, and glucose tolerance and SAT gene expression during the grazing season. A schematic presentation of the measurements made in the experiment III is given in Figure 3. Sixteen Finnhorse mares were divided into two groups and had 24 h a day free access to either cultivated high-yielding pasture or semi-natural grassland during the grazing season (May-September). The area of 4.5 ha cultivated pasture was divided into three equal paddocks and was dominated with three grass species: tall fescue (*Festuca arundinacea Schreb*.), timothy (*Phleum pratense L*.), and meadow fescue (*Festuca pratensis L*.). Two areas of semi-natural grassland were selected as trial areas. The first area consisted of 1.2 ha field and 6.5 ha of meadow/forest and the second area of 2.2 ha field and 3.6 ha meadow/forest, respectively. The experiment lasted from the end of May to the beginning of September (98 days).

3.2 Body and blood measurements

The complete protocols of the experimental measurements are described in detail within individual publications (I-III). In dairy cow experiments (I-II), body weights of cows were recorded on two consecutive days during the dry period and early lactation. All cows were body condition scored using the scoring system of Edmonson et al. (1989) throughout the experiments in conjunction with weighing. In the experiment III with mares, body weight, body condition score using the Henneke scoring system (Henneke et al., 1983) and waist circumference were measured. Subcutaneous fat thickness at neck and tailhead was measured using an Aloka SSD-500 ultrasound scanner with a 3.5 MHz transducer (III).

Blood samples were collected at 10.25 ± 5 d or 14.3 ± 4.98 d (mean ± SD) before the actual calving day, from the milk vein in the experiments I and II, respectively. After parturition, blood samples were collected from the coccygeal blood vessels at d 1 and 7 (±1) postpartum in the experiments I and II. The plasma concentrations of NEFA and glucose were analyzed as described by Salin et al. (2012). Plasma BHBA concentrations were measured spectrophotometrically. Plasma glucagon concentration was analyzed using radioimmunoassay. Insulin was measured by bovine-specific ELISA in the experiment I and as described by Salin et al. (2012) in the experiment II.

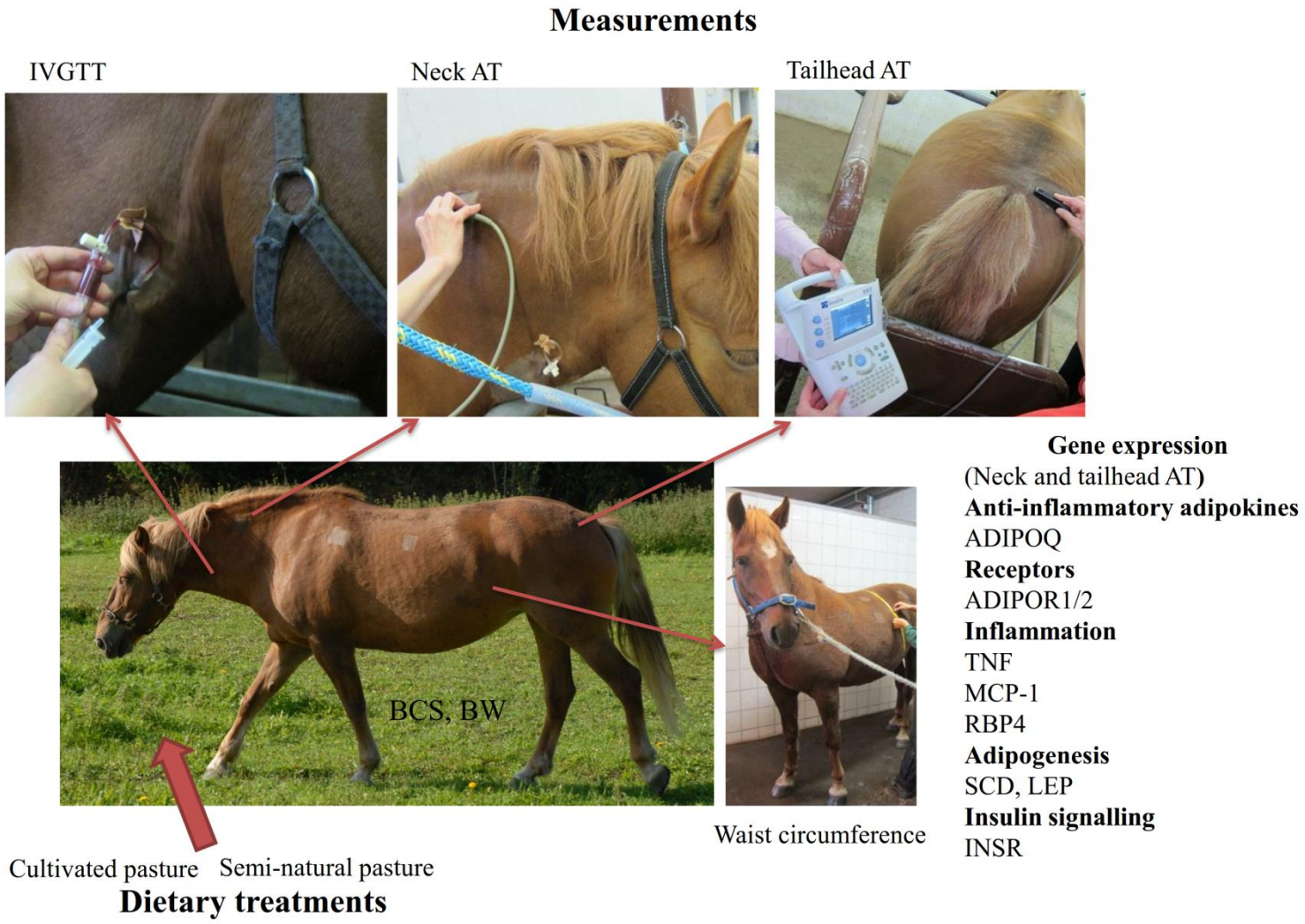

Figure 3. A schematic presentation of the measurements made in the experiment III. Photos were taken by Kari Elo and Susanna Särkijärvi.

In the experiment III, IVGTT was conducted at the beginning and at the end of the grazing season. After 12 h fasting, in the morning, mares received a jugular intravenous infusion of 0.3 g of glucose/kg body weight. During the 3-h duration of the IVGTT, venous blood samples were collected at -10, -5, 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 25, 30, 40, 50, 60, 70, 80, 90, 120, 150 and 180 min relative to the start of the glucose infusion. Plasma glucose, insulin, and NEFA responses to IVGTT were determined as the net incremental area under the response curve (AUC180; mmol/L × min for glucose and NEFA; μIU/mL × min for insulin) during the 180 min of IVGTT. Basal concentrations were defined as the mean concentration of blood samples taken before glucose infusion. The peaks of glucose and insulin, and the nadir of NEFA concentrations were determined. The clearance rate (CR180; %/min) of metabolites during IVGTT was calculated using PROC NLIN of SAS. Exponential curves for the calculation of clearance rate for glucose and NEFA concentrations during IVGTT were fitted using the equations described by Salin et al. (2012). In addition, the results of the IVGTT for each horse were analyzed according to the minimal model of glucose and insulin dynamics (Boston et al., 2003).

3.3 Tissue biopsies and gene expression analyses

Experimental procedures for tissue biopsies and gene expression analyses were described in detail in individual publications (I-III). Briefly, in the experiments I and II, tailhead SAT and liver tissue samples were taken during the transition period at the same day of blood sampling. In the experiment I, hepatic TAG concentration was measured by ultrahigh-performance liquid chromatography-mass spectrometry as described by Nygren et al. (2011), while in the experiment II, hepatic TAG was measured enzymatically and total lipids content was measured gravimetrically as described by Starke et al. (2010). In the experiment III, neck and tailhead SAT were collected in May and September from the same area where the fat thickness was measured, but on the other side of the mare. Total RNA was extracted from the liver tissue samples using RNeasy Mini Kit and from SAT samples using RNeasy Lipid Tissue Kit according to manufacturer's instructions. Determination of RNA quantity and quality was performed by measuring the absorbance of RNA extracts at 260 nm and 280 nm wavelengths using a NanoDrop ND-1000 spectrophotometer. Quality of RNA was assessed using RNA integrity number (RIN) on an Agilent Bioanalyzer 2100 chip electrophoresis system with Agilent RNA 6000 Nano Kit according to manufacturer’s instructions. First-strand cDNA was synthesized with

Anchored-Oligo (dT)18 primer using Transcriptor First Strand cDNA Synthesis Kit in a 20 µl reaction according to manufacturer's instructions. The cDNA was diluted to 1:8 with DNase/RNase free water in the experiments I and II and to 1:4 in the experiment III.

Primers for the studied genes were designed using the online Primer3 software program (Rozen and Skaletzky, 2000). The uniqueness of primer sequences was established using BLAST search tool and NCBI GenBank sequence database. The DNA sequences for primers, GenBank accession numbers for DNA sequences used, PCR product lengths, analyzed tissue(s) and references for published primers are presented in publications I-III. The internal control gene used in the experiments I and II was eukaryotic translation initiation factor 3 subunit K (*EIF3K*). *EIF3K* has been shown to be one of the most stable internal control genes in bovine liver and AT (Saremi et al., 2012; Bonnet et al., 2013). The internal control gene used in the experiment III was mitochondrial ribosomal protein L39 (*MRPL39*). *MRPL39* has been reported to be one of the stable internal control genes in AT of horses (Bruynsteen et al., 2013). The expression stability was tested using NormFinder software (Andersen et al., 2004), and the mRNA abundance of internal control gene was stable across groups and time points. However, it has been suggested that the geometric average of more than one internal control gene is the ideal for normalization of quantitative Real-Time PCR data (Saremi et al., 2012; Bonnet et al., 2013). Using only one internal control gene is not the ideal approach for calculation of normalization factor, although it gives results that are accurate enough for meaningful comparisons between treatment groups and time points.

The qPCR reactions were performed in optical 384-well plates using LightCycler 480 instrument (Roche Diagnostics GmbH, Mannheim, Germany). Reactions with a total volume of 10 µl were prepared by mixing 2.5 µl of diluted first-stand cDNA and 7.5 µl of master-mix using EpMotion automated pipetting system. The master-mix composed 5 µl 2× SYBR Green master mix, 0.5 µl (5 pmol/µl) each of forward and reverse primers (except for *MCP-1* in the experiment III, it was 20 pmol/µl each for forward and reverse primers), and 1.5 µl DNase/RNase free water. Each cDNA sample was run in quadruplicate for each gene. The temperature profile of the qPCR was as follows: initial denaturation step for 5 min at 95 °C, followed by 45 amplification cycles for 20 sec at 95 °C, 20 sec at 60 °C, and 20 sec at 72 °C. A melting curve analysis was run in the end of the PCR program. The mRNA abundance was calculated relative to the expression of the

internal control gene, i.e. the difference between the average Ct of target gene replicates and the average Ct of internal control gene replicates was presented as delta cycle threshold (constant-ΔCt). The mRNA abundance data (I-III) were presented as $\log_2$-transformation of ΔCt (constant-ΔCt).

3.4 Statistical analysis

Statistical computations were performed using SAS (SAS Institute Inc., Cary, NC, USA, release 9.2/9.3). Prior to statistical analysis, residuals of all data were checked for normality and outliers using the MIXED and UNIVARIATE procedures of SAS. Data that were not normally distributed were $\log_2$-transformed to create a normal distribution of data. If data were not normally distributed after $\log_2$-transformation, data points with highest studentized residuals were considered outliers. In dairy cow experiments (I-II), final data were analyzed by repeated measures ANOVA using model that included fixed effects of treatment, time, and the interaction between treatment and time, and random effect of block and the interaction between block and time. When the interaction of treatment and time was significant, differences between treatments at each time point were tested for significance using the slice option of the SAS MIXED procedure. In the experiment III, final data were analyzed using a model that included a fixed effect of treatment and a random effect of pair. May (pre-grazing) values were used as covariates when analyzing September data. Data with two time points (hepatic TAG concentrations in the experiment I and changes over time in the experiment III) were analyzed using a model that included fixed effects of treatment, time and the interaction between treatment and time, and a random effect of block/pair. The statistical significance was declared at $P < 0.05$ and trend at $0.05 \leq P < 0.15$. Spearman rank correlation coefficients were calculated using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) to identify significant correlations ($P < 0.05$).

Table 1. Summary of the experiments.

Exp.	Number of animals	Exp. period	Treatments	Species	Objectives
I	16	44 d prepartum to d 9 postpartum	• Restricted feeding of grass silage (99 MJ/d ME) • High energy diet (141 MJ/d ME) during first 3 wk of the dry period + decreasing energy allowance during the last 3 wk to 99 MJ/d ME by parturition	Multiparous Finnish Ayrshire dairy cows	To study the effects of overfeeding grass silage combined with gradually decreasing energy allowance by parturition on the liver and SAT gene expression during the transition period
II	16	58 d prepartum to d 7 postpartum	• Controlled energy diet (109 MJ/d ME) • High energy diet (144 MJ/d ME) during the dry period	Multiparous Finnish Ayrshire dairy cows	To evaluate the effects of *ad libitum* feeding of grass silage throughout the dry period on the liver gene expression and SAT gene expression during the periparturient period
III	16	From the end of May to the beginning of September (98 d)	• Semi-natural grassland pasture • Cultivated high-yielding pasture	Non-pregnant Finnhorse mares	To assess the effect of high pasture allowance on fat deposition, responses during IVGTT and SAT gene expressions during the grazing season.

Exp., experiment; ME, metabolizable energy; SAT, subcutaneous adipose tissue; IVGTT, intravenous glucose tolerance test.

4. RESULTS AND DISCUSSION

4.1 Dietary energy, body condition and body reserves in dairy cows and mares

Excess dietary energy intake causes obesity or localized adiposity in different species. Obesity results from the imbalance between energy intake and energy expenditure. Level of feeding and dietary composition have been reported to affect body condition both in cows (Dann et al., 2006; Ji et al., 2012) and equine (Hoffman et al., 2003; Longland et al., 2011a, b). Obese animals are at a greater risk for developing health-related problems both in dairy cows (Dann et al., 2006; Douglas et al., 2006) and in horses (Treiber et al., 2006; Carter, 2008).

In the experiments I and II, overfed cows had higher total DMI, ME intake and ME balance than control cows. In the experiment II, DMI and ME intake of the TMR group was effectively controlled during the entire dry period by the inclusion of wheat straw in TMR. Inclusion of bulky lower quality roughage during the dry period, such as wheat straw, has been successfully used to limit energy intake and BCS gain prepartum (Dann et al., 2006; Janovick and Drackley, 2010; Ji et al., 2012).

Pasture intake of horses typically ranges from 2-2.5% of BW as DM (95-118 g DM/kg$^{0.75}$ for a 500 kg horse; NRC, 2007); even though pasture intake has been reported to be as high as 5.2% of BW (Smith et al., 2007). Estimation of long-term pasture intake can be achieved by measuring changes in body weight over a number of weeks from known digestible energy requirements for maintenance, activity and gain (Longland et al., 2011b). Feed values of the semi-natural grassland pastures during the grazing season (June-September) were adequate to meet the nutrient requirements of Finnhorse mares (Herzon et al., 2014). Grazing on a high-yielding cultivated pasture easily induces weight gain in horses and the DMI rate can be as high as 4% BW basis (Longland and Byrd, 2006).

In the experiment I, there were no differences between the groups for BW and BCS changes during the dry period. However in the experiment II, greater BW gain in cows fed *ad libitum* grass silage prepartum and a relatively small difference in BCS change between the groups during the dry period was observed. These results suggest that *ad libitum* feeding of grass silage during the dry period may increase the susceptibility for

visceral AT accumulation. In non-lactating, non-pregnant dairy cows, overfeeding energy for 8 weeks resulted in higher BW and internal fat mass without a noticeable increase in BCS (Drackley et al., 2014). In the experiment III, horses grazed on cultivated high-yielding pasture had greater BW and waist circumference, and higher median of BCS compared to horses grazed on semi-natural grassland. Frank et al. (2006) and Vick et al. (2007) have defined horses with BCS ≥ 7 as obese. These findings (III) suggest that CG mares were overweight at the end of the grazing season and may have accumulated visceral fat, in addition to subcutaneous fat.

4.2 Adipose tissue gene expression in dairy cows and mares

Adipose tissue is a dynamic endocrine organ, secreting hormones and inflammatory mediators which have been implicated in the inflammatory response and IR in dairy cows (Vernon, 2005; Ji et al., 2012; Khan et al., 2013) and equine (Vick et al., 2007; Burns et al., 2010; Ungru et al., 2012). Key adipokines and other substances secreted or produced by AT and their relations to obesity, IR and inflammation based on human and laboratory animals studies are presented in Table 2.

Studies included in publications I-III investigated the effects of high vs. control energy intake on the mRNA expression of key genes that play important roles in major metabolic pathways in SAT. These key adipokines, enzymes and receptors were primarily studied at the transcriptional level, which does not necessarily reflect the protein level or activity. Measuring the mRNA expression is technically the most feasible and accurate approach for small tissue samples.

Table 2. Key adipokines and other substances secreted or produced by adipose tissue and their relations to obesity, insulin resistance and inflammation (based on human and laboratory animals studies).

Protein	Effects on	Relation to			Reference
		Obesity	IR	Inflammation	
Adipokines					
ADIPOQ	Glucose and lipid metabolism, insulin sensitivity and anti-inflammatory function	↓	↓	↓	Ronti et al. (2006); de Luca and Olefsky (2008)
IL6	Pro-inflammatory response, lipid metabolism and energy homeostasis	↑	↑	↑	Ronti et al. (2006); de Luca and Olefsky (2008)
Leptin	Food intake, immunity, insulin sensitivity and inflammation	↑	↑	↑	Ronti et al. (2006); de Luca and Olefsky (2008)
MCP-1	Pro-inflammatory response	↑	↑	↑	Ronti et al. (2006); de Luca and Olefsky (2008)
TNFα	Pro-inflammatory response and antagonism of insulin signalling	↑	↑	↑	Ronti et al. (2006); de Luca and Olefsky (2008)
Resistin	Inflammation and insulin sensitivity	↑	↑	↑	Ronti et al. (2006); de Luca and Olefsky (2008)
RBP4	Lipid and glucose metabolism, insulin sensitivity and inflammation	↑	↑	↑	Yang et al. (2005); Norseen et al. (2012)
Receptors					
PPARG	Lipid metabolism and insulin sensitivity	↓	↓	↓	Guilherme et al. (2008)
INSR	Insulin signalling and glucose homeostasis	↓	↓	↓	de Luca and Olefsky (2008)
IRS1	Insulin signal transmission and glucose homeostasis	↓	↓	↓	de Luca and Olefsky (2008)
Enzymes					
HSL	Lipid metabolism (lipolysis)	↑	↑	↑	Guilherme et al. (2008)
LPL	Lipid metabolism (fatty acid uptake)	↓	↓	↓	Guilherme et al. (2008)
PCK1	Lipid metabolism (glyceroneogenesis)	↓	↓	↓	Guilherme et al. (2008)
SCD	Lipid metabolism (fatty acid desaturation)	↑	↑	↑	Liu et al. (2010)

↑, increase; ↓, decrease; ADIPOQ, adiponectin; IL6, interleukin 6; MCP-1, monocyte chemoattractant protein-1; TNFα, tumor necrosis factor-alpha; RBP4, retinol binding protein 4; PPARG, peroxisome proliferator activated receptor-gamma; INSR, insulin receptor; IRS1, insulin receptor substrate 1; LPL, lipoprotein lipase; HSL, hormone sensitive lipase;; PCK1, cytosolic phosphoenolpyruvate carboxykinase 1; SCD, stearoyl-CoA desaturase; IR, insulin resistance.

4.2.1 Anti-inflammatory adipocytokines and insulin signalling

Adiponectin is known to influence fatty acid metabolism and glucose homeostasis (Kadowaki and Yamauchi, 2005). Adiponectin exerts anti-inflammatory properties by reducing the secretion of pro-inflammatory cytokines as shown in studies with LPS challenge (Thakur et al., 2005; Tsang et al., 2011). However, the mechanism underlying these effects remains incompletely understood. Adiponectin induces its effects through interaction with its specific cell surface receptors, adiponectin receptor 1 and 2 (ADIPOR1/R2) (Kadowaki et al., 2006). Circulating ADIPOQ has been shown to be inversely correlated with BCS both in horses (Kearns et al., 2006; Gordon et al., 2007) and cows (Singh et al., 2014).

Excess dietary energy intake and increased BCS did not affect the mRNA expression of *ADIPOQ* in dairy cows (I-II). There were no differences in the mRNA expression of *ADIPOR1/R2* in the experiment I, while a down-regulation of *ADIPOR1/R2* was noticed in the overfed cows of the experiment II. These results suggest that overfed cows in the experiment II may have had lower insulin sensitivity in SAT compared to TMR control cows. Ji et al. (2012) reported up-regulation of *ADIPOQ* early postpartum in overfed cows. Breed, diet and body condition may have been attributed to the inconsistencies between the studies (Ji et al., 2012; I and II). The lack of changes in the expression of *ADIPOQ* and its receptors from pregnancy to early lactation in the experiments I and II was in line with some of the earlier studies (Sadri et al., 2011; Giesy et al., 2012), but in contrast to more recent studies (Ji et al., 2012; Khan et al., 2013; Saremi et al., 2014). Differences between breeds and the timing of biopsy may have contributed to the variation of results (Sadri et al., 2011; Giesy et al., 2012; Ji et al., 2012; Khan et al., 2013; Saremi et al., 2014; I; II).

In the experiment with mares (III), grazing on high-yielding pasture and increased BCS did not affect the mRNA abundance of *ADIPOQ* and its receptors in both neck and tailhead SAT. In ponies, with body weight reduction program blood ADIPOQ concentration was increased, but its mRNA expression in tailhead SAT was not affected (Ungru et al., 2012). There was up-regulation of *ADIPOQ* and its receptors after the end of the grazing season in tailhead SAT but not in neck SAT. This suggests that insulin sensitivity and fat deposition were greater in tailhead SAT than in neck SAT. In cultured adipocytes, Fu et al. (2005) observed that over-expression of *ADIPOQ* accelerated

differentiation of adipocyte and augmented lipid accumulation and insulin-responsive glucose transport. Bruynsteen et al. (2013) suggested that AT depots at loin and tailhead are more stimulated to differentiate pre-adipocytes into adipocytes than other fat depots in horses.

In the experiments with dairy cows (I-II) and mares (III), *ADIPOQ* mRNA correlated positively with its receptors in SAT, and mRNA abundances of *ADIPOQ* receptors were positively correlated with each other. In mice, Tsuchida et al. (2004) reported that the expressions of *ADIPOR1*/2 were correlated with ADIPOQ sensitivity. In dairy cows, Lemor et al. (2009) observed that *ADIPOR1* expression in AT was positively correlated with *ADIPOR2* mRNA. Therefore, the observed positive correlations between *ADIPOQ* and its receptors in the experiments I-III suggest a concurrent regulation of *ADIPOQ* and its receptors, at least at transcriptional level.

Insulin receptor substrates are proteins that take the first intracellular step to mediate insulin signaling. Insulin receptor substrate 1 (*IRS1*) is known to be responsible for insulin-induced metabolic actions, including glucose homeostasis (Saltiel and Kahn, 2001). Overfeeding of energy did not affect the mRNA expression of *IRS1* in dairy cows (I). In the experiment I, the observed higher insulin concentrations in overfed cows during the dry period were not large enough to induce a significant effect on *IRS1* mRNA expression. In agreement with the results of the experiment I, Ji et al. (2012) did not observe any differences in the mRNA expression of *IRS1* in SAT between overfed and control cows during the transition period. Lack of difference in *IRS1* expression around parturition in Ji et al. (2012) and in the experiment I is in line with the idea that a defect in post-translational modification of *IRS1* is a major mechanism aggravating IR in late pregnancy (Vernon and Taylor, 1988).

In the experiment with mares (III), there was no difference in the mRNA expression of *INSR* between CG and NG mares in September. This may indicate that the observed higher basal insulin concentration in CG mares in September was not large enough to persuade significant differences in *INSR* mRNA expression. The *INSR* mRNA abundance was down-regulated in September compared to May in both groups. This may indicate a negative feedback mechanism of insulin on the insulin signaling pathway in SAT (Suagee et al., 2011).

4.2.2 Inflammatory adipocytokines

Obesity is a pro-inflammatory state where lymphocytes and macrophages are recruited into AT in response to higher monocyte chemoattractant protein-1 (MCP-1), and both lymphocytes and macrophages may contribute to elevated circulating levels of pro-inflammatory cytokines such as TNFα and interleukin 6 (IL6) (Tateya et al., 2010; Makki et al., 2013). Both TNFα and IL6 are potent pro-inflammatory cytokines, and their circulating levels are positively related to IR (Bruun et al., 2003). Tumor necrosis factor-α can induce IR directly via activation of a number of protein kinases that phosphorylate the IRS proteins (e.g. Serine/Threonine phosphorylation) and then induce negative effect on insulin signalling in a number of ways (Boura-Halfon and Zick, 2009), or indirectly by inhibiting differentiation of adipocytes and lipid metabolism (Xu et al., 1999). The release of IL6 is increased by activation of TNFα, and IL6 has been shown to inhibit insulin signalling in cultured murine adipocytes (Rotter et al., 2003). IL6 also increases lipolysis (van Hall et al., 2003) and down-regulates the expression of other cytokines such as *ADIPOQ* (Sopasakis et al., 2004).

In the experiment I, there was no effect of prepartal energy overfeeding on the mRNA abundance of *IL6*, suggesting a weak association between this adipokine and the control of insulin responsiveness or IR in AT during the transition period. These results were in line with Ji (2012) who observed no difference in *IL6* mRNA between overfed and control cows. A trend for higher mRNA abundance of *TNF* in overfed cows compared to control cows at d 9 after parturition (I) may suggest aggravated inflammation and attenuated insulin sensitivity in SAT. Winkelman et al. (2008) reported no differences in the plasma TNFα levels between limited and *ad libitum* feeding of dairy cows pre- and postpartum.

In the experiment with mares, there was no difference in *TNF* mRNA expression between CG and NG mares (III). The mRNA abundance of *TNF* was not affected by obesity-associated IR (Burns et al., 2010) or IR status (Carter, 2008) in horses. However, obesity and IR have been shown to be associated with higher plasma TNFα concentrations in horses (Vick et al., 2007) and ponies (Treiber et al., 2009). Results of the experiment III with mares suggest that the level of BW and BCS gains in CG mares during the grazing season may not be associated with low-grade inflammation in SAT. Taken together, higher plasma level of TNFα in obesity or IR (Vick et al., 2007; Treiber et al., 2009) and

absence of differences at transcriptional level in SAT (Burns et al., 2010; Ungru et al., 2012; III) suggest that visceral AT may influence TNFα secretion in cases of obesity-associated IR in equine.

The mRNA expression of *MCP-1* in AT was up-regulated and directly correlated with adiposity in obese human subjects (Christiansen et al., 2005) and mice (Tateya et al., 2010). However, in the experiment III with mares, *MCP-1* mRNA was not different between CG and NG mares in SAT at the end of the grazing season, and it was down-regulated in September compared to May. In non-obese and over-conditioned horses, *MCP-1* mRNA expression was not altered by insulin sensitivity or difference between fat depots (Burns et al., 2010). These findings may indicate species-related differences in *MCP-1* expression or alternatively inflammation in AT may not occur until the onset of obesity or after a prolonged period of obesity.

Horses demonstrate a seasonal up-regulation of the hypothalamic-pituitary axis, with higher circulating concentration of alpha-melanocyte stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH) in autumn than in winter and spring (McFarlane et al., 2004; Frank et al., 2010a). Alpha-MSH is known to inhibit the production of pro-inflammatory cytokines and chemokines contributing to inflammation (Lipton and Catania, 1997), and may influence long-term regulation of feed intake and energy balance (Schuhler and Ebling, 2006). It is possible that the observed down-regulation of *MCP-1* gene expression at the end of the grazing season (III) may contribute to seasonal adaptations of horses to winter.

4.2.3 Adipose tissue lipid metabolism

4.2.3.1 Lipogenesis

Adipose tissue is the predominant site of lipogenesis (Ingle et al., 1972; Bergen and Mersmann, 2005). Peroxisome proliferator-activated receptor gamma is one of the most important nuclear transcription receptor that plays a critical role in adipogenesis and insulin sensitivity of AT (Bionaz et al., 2013). Peroxisome proliferator-activated receptor gamma can regulate the expression of several lipogenic genes such as cytosolic phosphoenolpyruvate carboxykinase 1 (*PCK1)* (Tontonoz et al., 1995), lipoprotein lipase (*LPL)* (Schoonjans et al., 1996) and stearoyl-CoA desaturase *(SCD)* (Risérus et al., 2005).

Excess energy intake prepartum down-regulated the transcriptional activity of *LPL* and *SCD* in overfed cows early postpartum (II); however, in the experiment I, down-regulation of *SCD* at d 1 and up-regulation at d 9 postpartum in the HIGH cows was observed. These findings were in line with Ji et al. (2012) and suggest that overfeeding energy throughout the dry period (II) induced more deeply decreased lipogenesis early postpartum compared to the experiment I. Ji et al. (2012) suggested that down-regulation of lipogenesis-related genes in the overfed cows may contribute to exacerbated IR early postpartum. However, the expression of *PPARG* did not differ between treatments over time (I-II), which was in contrast to Ji et al. (2012). The relative changes in the mRNA expression of *PPARG* target genes (*LPL*, *SCD*, *PCK1*) in response to prepartal energy or lactation could be taken as indicators for the activity of *PPARG* in AT. van Dorland et al. (2009) and Graugnard et al. (2013) reported changes in the expression patterns of *PPARA* target genes in the liver without noticeable changes in this nuclear receptor. Bionaz et al. (2013) suggested that target genes of PPARs can be used as proxies for evaluating the activation of PPARs.

Adipose tissue metabolism is usually in catabolic mode during early lactation and is characterized by reduced fatty acid synthesis, fatty acid uptake and esterification (Sumner and McNamara, 2007; Sumner-Thomson et al., 2011). A coordinated down-regulation of genes involved in fatty acid uptake (*LPL*, I and II), fatty acid desaturation (*SCD*, I and II), and glyceroneogenesis (*PCK1*, I) was observed postpartum compared to prepartum, which may attribute to exacerbated IR (Ji et al., 2012).

In the experiment with mares (III), there was no difference in the mRNA expression of *SCD* in SAT between CG and NG mares. However, the mRNA abundance of *SCD* was increased in tailhead, but not in neck SAT during the grazing season (III), in line with greater fat thickness in tailhead compared to neck during the grazing season, and therefore lipid accumulation may differ between fat depots in horses.

4.2.3.2 Adipokines

Leptin (LEP) regulates appetite, energy homeostasis and adipogenesis (Ingvartsen and Boisclair, 2001). Leptin is secreted primarily by adipocytes, and its blood concentration has been positively correlated with body fat mass in ruminants (Ehrhardt et al., 2000; Kokkonen et al., 2005) and horses (Buff et al., 2002; Kearns et al., 2006). A recent study

by Ji et al. (2014) reported up-regulation of *LEP* gene expression in overfed non-pregnant, non-lactating dairy cows. In the experiment I with dairy cows, dietary treatments did not affect *LEP* gene expression; however, in the experiment II, *LEP* was down-regulated in overfed cows early postpartum compared to control cows. Lower *LEP* expression early postpartum in both groups and more notably in the overfed group (II) may support the concomitant retrieval of DMI and energy balance (Roche et al., 2013). The down-regulation of *LEP* during the periparturient period agreed with previous studies showing a decrease of plasma LEP (Block et al., 2001; Kokkonen et al., 2005) or *LEP* expression (Saremi et al., 2014), and it was also consistent with the decline of plasma insulin during the transition period (Block et al., 2001).

In the experiment with mares (III), the mRNA expression of *LEP* was not different between CG and NG mares in both neck and tailhead SAT. Blood LEP was reported to increase, along with BW and body fat, in young and mature Thoroughbred mares from July to September (Fitzgerald and McManus, 2000). Another study, however, suggested that factors other than fatness determine the relative levels of LEP secretion in horses with similar adiposity (Gentry et al., 2002). The down-regulation of *LEP* mRNA in neck SAT of mares in September (III) may be in part due to the rise of α-MSH in preparation for winter. In cultured rat adipocytes, administration of α-MSH inhibited LEP secretion and gene expression (Hoggard et al., 2004). Thus further studies are required to assess the relationship between seasonal variation of α-MSH and blood LEP concentration and *LEP* gene expression in SAT.

Retinol binding protein (RBP4) is a cytokine secreted by adipocytes, and higher blood levels of RBP4 were shown in adiposity and type 2 diabetes (Klöting et al., 2007; Lee et al., 2007). Insulin-resistant mice were shown to have higher serum RBP4 and mRNA expression of *RBP4* in AT (Yang et al., 2005). Further, Yang et al. (2005) demonstrated that changes in AT RBP4 can affect systemic insulin sensitivity and glucose homeostasis (Yang et al., 2005). Excess energy intake did not affect the expression of *RBP4* in SAT in transition dairy cows (I) or in grazing mares (III). *RBP4* expression decreased in SAT of the studied animals after either parturition (I) or the grazing season (III). There is very little information regarding transcriptional activity of *RBP4* and its role in bovine and equine SAT. In the IR states of humans, visceral fat was reported as the major source of RBP4 (Klöting et al., 2007; Lee et al., 2007). In bovine, Locher et al. (2009) reported that

RBP4 expression was greater in visceral fat than in SAT. In ponies, the study by Ungru et al. (2012) reported a significant decrease in blood RBP4 with body weight reduction program, but there was no difference in its mRNA expression in tailhead SAT between insulin sensitive and insulin resistant ponies. Additionally, Ungru et al. (2012) suggested that serum RBP4 was closely related to adiposity.

Resistin (RETN) is considered an important factor underlying adiposity-associated IR in mice (Steppan et al., 2001), and Komatsu et al. (2003) reported that the expression of *RETN* differed in bovine AT between non-lactating and lactating cows. In the experiment I with dairy cows, there was a trend for lower mRNA expression of *RETN* in HIGH cows than in control cows. This may indicate that the degree of energy overfeeding achieved in the experiment I during the dry period may not aggravate IR in SAT. In addition, the observed up-regulation of *RETN* early postpartum compared to prepartum in both groups may contribute to lipid mobilization and decreased insulin sensitivity in SAT during early lactation. These results were in line with Komatsu et al. (2003) and Reverchon et al. (2014) who reported that *RETN* mRNA expression in SAT increased in cows during the early phase of lactation.

4.2.3.3 Lipolysis

Cows undergo a natural NEB after parturition, and lipolysis is the main pathway by which cows can compensate for their energy deficits. Earlier it was thought that hormone sensitive lipase (HSL/*LIPE*) is the rate limiting enzyme in lipolysis (Vaughan et al., 1964). Recently, additional proteins have been reported to be critical regulators of lipolysis in AT, such as perilipin and patatin-like phospholipase domain containing 2, also known as cytosolic adipose triglyceride lipase (ATGL/*PNPLA2*) (Zimmermann et al., 2004; Khan et al., 2013). Excess prepartal energy intake in the experiments I and II did not affect the mRNA abundance of *LIPE* during the periparturient period. These results (I-II) were in contrast to Sumner and McNamara (2007) and Khan et al. (2013). The discrepancies between the studies may be due to different time scale. Increased HSL phosphorylation in SAT in early lactation was observed (Koltes and Spurlock, 2011), and AT of fatter cows tended to show higher in vitro lipolytic responses to added norepinephrine than that of leaner cows (Kokkonen et al., 2005). Taken together, the activity of HSL may be regulated post-translationally (Loor, 2010; Khan et al., 2013); however, in the current studies, post-translational regulation of HSL was not measured.

4.2.3.4 Fatty acid β-oxidation

Mitochondrial carnitine palmitoyltransferase 1A (CPT1A) regulates the translocation of long chain fatty acids from the cytoplasm into the mitochondria, and hence fatty acid β-oxidation. *CPT1A* protects adipocytes from fatty acid-induced IR and inflammation (Gao et al., 2011) and may be an indicative of increased use of fatty acids as energy in AT (Elis et al., 2013). Nevertheless, the functional roles of *CPT1A* in adipocytes during the transition period in dairy cows are not fully elucidated.

4.3 Liver gene expression in dairy cows

In Europe and North America, milk yield of dairy cows has increased dramatically due to advanced feeding and management strategies. However, this increase has been accompanied by higher incidences of metabolic diseases (Heuer et al., 1999; Fleischer et al., 2001) and reproductive problems (Lucy et al. 2001), and as a result the longevity of dairy cows has shortened steadily (Gröhn et al., 1998). Improving cow's longevity by decreasing the rate of culling has both economic (Essl, 1998) and welfare benefits (Loor et al., 2013). During the last two decades, research has focused on transition cow nutrition and management concerning the nutritional physiology of dairy cow to improve transition health and performance (Grummer, 1995; Grummer et al., 2004; Janovick et al., 2011). Successful metabolic adaptations of the liver lipid and glucose metabolism during the transition period are essential for optimal health and production of dairy cows (Drackley et al., 2001; van Knegsel et al., 2014). Effects of prepartal energy intake on hepatic gene expressions of dairy cows are presented in Table 3.

4.3.1 Liver glucose metabolism

In ruminants, dietary carbohydrates are degraded to short-chain fatty acids by the action of microbial fermentation, and only 10% of the cow's glucose requirement is absorbed from the intestine (Aschenbach et al., 2010). This means that dairy cows have to depend largely on hepatic gluconeogenic capacity to meet their glucose requirements (Bell and Bauman, 1997). Therefore, efficient gluconeogenesis is crucial for maintaining a sufficient supply of glucose to the mammary gland (Bell and Bauman, 1997). About 85% of the glucose entry resulting from hepatic gluconeogenesis may be used by the mammary gland for milk production in lactating dairy cows (Annison et al., 1974). Accordingly, the regulation of gluconeogenesis is an area of interest in the milk

production and health status of dairy cows. In the liver, glucose production is controlled by substrate availability, the metabolic capacity of hepatocytes to synthesize glucose and hormonal status of dairy cows (Aschenbach et al., 2010).

Table 3. Effects of overfeeding energy during the dry period on the expressions of key genes related to liver glucose and lipid metabolisms, insulin and growth hormone signalling, and inflammation in multiparous dairy cows.

Prepartal energy	Time RTC	Genes	Breed	References
Corn and alfalfa silage either: • Restricted (80% of NER) • *Ad libitum* moderate energy diet (> 140% NER)	-65, -30, -14, 1, 14, 28 & 49 d	↑*DGAT1* pp ↑*NFKB1* pre.p &↓pp ↓*CPT1A* & *PC*	Holstein	Loor et al. (2006)
• *Ad libitum* TMR (100% of NER), wheat straw ~41.9% of TMR • Moderate energy diet (>140% NER), corn silage ~50.3% of total.	-14, 7, 14 & 30 d	↑*CPT1A* pp. ↓*PC* pre.p ↑*IGF*-1 pre.p ↑*PCK1 pp* ↔*PPARA*	Holstein	Khan et al. (2014)
• Restricted feeding of grass silage (~100% of MER). • *Ad libitum* grass silage (~140% MER) during far-off + decrease to ~100% MER by parturition.	-10, 1 & 9 d	↓*PC*, *PCK1* & *G6PC* ↓*CPT1A* ↔*RBP4, IRS1 & NFKB1*	Finnish Ayrshire	I
Ad libitum either: • Grass silage (~140% MER). • TMR (55% grass silage, 40% wheat straw and 5% rapeseed meal (~100% of MER).	-14, 1 & 7 d	↔*PC*, *G6PC* & *SLC2A2* ↓*PCK1* ↔*CPT1A*, *PPARA*, *SLC22A5* & *DGAT1* ↔*IRS1* & *IGF-1*, *NFKB1*, *IL6* *RBP4*, *PPARG*	Finnish Ayrshire	II

Effect of prepartal energy intake on gene expression (↑, up-regulation; ↓, down-regulation; ↔, no changes). TMR, total mixed ration; NER, net energy requirement for pregnant dairy cows; MER, metabolizable energy requirement for pregnant dairy cows; RTC, relative to calving; pp, postpartum; pre. p, prepartum.

Propionate is the principle precursor for hepatic gluconeogenesis, but during the early lactation period propionate supply is not enough to meet the need of glucose synthesis due to insufficient DMI (Larsen and Kristensen, 2013). Thus, amino acids from diet or skeletal muscle breakdown and glycerol from lipolysis of AT can be used as substrates

for glucose synthesis to meet the cow's glucose demands during this period (Reynolds et al., 2003; Larsen and Kristensen, 2013). In the immediate postpartum period, the release of glucose from the liver seems to be relatively more dependent on lactate uptake (Reynolds et al., 2003; Larsen and Kristensen, 2013). Pyruvate carboxylase (PC) is required for the entry of lactate and gluconeogenic amino acids into the gluconeogenic pathway (Pilkis and Granner, 1992). Cytosolic phosphoenolpyruvate carboxykinase 1 (PCK1) has been established as a main rate-limiting enzyme involved in glucose production from propionate in dairy cows (Rukkwamsuk et al., 1999; Greenfield et al., 2000). Glucose-6-phosphatase plays a role in the dephosphorylation of glucose-6-phosphate and produces free glucose (van Schaftingen and Gerin, 2002).

It is generally acknowledged that insulin is a potent inhibitor of hepatic gluconeogenesis (Hayirli, 2006). Several studies confirmed the suppressing effect of insulin on hepatic gluconeogenesis (e.g. Bradford and Allen, 2005; Loor et al., 2006; Kreipe et al., 2011). However, the molecular mechanisms by which insulin influences gluconeogenic genes are not fully clarified. Drackley et al. (2001 and 2005) suggested that fat accumulation in the liver is potentially linked to impaired hepatic capacity to synthesize glucose. The activity (Rukkwamsuk et al., 1999; Murondoti et al., 2004) and the mRNA expression (Graber et al., 2010) of hepatic gluconeogenic enzymes have been reported to decrease with high liver fat content in dairy cows. However, Hammon et al. (2009) reported that the increased hepatic lipid content did not impair the expression of gluconeogenic genes in the liver of dairy cows.

In the experiment I, prepartal *ad libitum* feeding of grass silage for three weeks during early dry period combined with gradually decreased energy allowance during the next three weeks down-regulated the mRNA expression of *PC*, *PCK1* and *G6PC* during the transition period. However, in the experiment II, *ad libitum* feeding of grass silage during the dry period down-regulated only the expression of hepatic *PCK1* early postpartum, but there were no differences in the mRNA abundance of *PC* or *G6PC* during the transition period. In the experiments I and II, overfed cows tended to have higher plasma insulin concentration during the dry period, which may explain the down-regulation of hepatic gluconeogenic genes. Higher blood insulin concentration was shown to decrease hepatic mRNA expression of *PC* (Loor et al., 2006).

In the experiment I, an increase in the mRNA abundance of *PC* postpartum in comparison to prepartum was observed, but this increase was not noticed in the experiment II. Elevated enzyme activity or mRNA level of *PC* postpartum has been reported by Greenfield et al. (2000), Murondoti et al. (2004), Loor et al. (2006) and Khan et al. (2014). This increase in the activity or mRNA of *PC* has been suggested as an adaptive adjustment in the liver to meet mammary glucose requirements for milk production (Greenfield et al., 2000; Loor et al., 2006). Immediately after parturition, increased activity of PC allows a greater entry of glucogenic precursors, including lactate and gluconeogenic amino acids via pyruvate to the Krebs cycle (Larsen and Kristensen, 2013).

PC is known to be regulated by insulin/glucagon ratio and NEB (Greenfield et al., 2000; Loor et al., 2006; Aschenbach et al., 2010; Akbar et al., 2013). Greater glucagon/insulin ratio (0.80 vs. 0.70 mol/mol) and NEB (-81 vs. -55 MJ ME/d) at lactation week 1 in the experiment I compared to the experiment II may explain the differences in *PC* expression patterns between the two experiments. The decreased ruminal propionate production due to feeding grass silage diet and the absence of concentrate in the prepartal diet prior to the first biopsy (-14 d) in the experiment II may have accelerated the utilization of lactate and alanine (Vanhatalo et al., 2003), and thus may have enhanced the gene expression of *PC* prepartum in the experiment II. In support of this, prepartal plasma glucose concentration in the experiment I was higher than in the experiment II (4.25 vs. 3.6 mmol/L). The lack of time-related change in *PCK1* gene expression in the experiments I and II was in agreement with previous findings (Greenfield et al., 2000; Murondoti et al., 2004; Khan et al., 2014). It has been suggested that the expression of *PCK1* mRNA is related to the amount of glucogenic precursors provided from ruminal fermentation of ingested feeds (Larsen and Kristensen, 2013). Taken together, the time patterns of *PC* and *PCK1* gene expression in the experiments I and II are most likely coordinated by the relative availability of glucogenic precursors (Aschenbach et al., 2010; Larsen and Kristensen, 2013).

In the experiment II, there was no treatment or time- related changes in the expression of glucose transporter 2 *(SLC2A2)*. SLC2A2 is the bidirectional glucose transporter in the plasma membrane (Zhao et al., 1993). Hepatic *SLC2A2* has been used previously in dairy cows to evaluate glucogenic status, in particular the liver release of glucose (Hammon et

al., 2009; Goselink et al., 2013). Goselink et al. (2013) reported that rumen-protected choline supplemented diet increased the expression of hepatic *SLC2A2*, but did not influence milk yield or lactose yield. Mach et al. (2013) observed an increase in the expression of *SLC2A2* in cows fed diet supplemented with linseed compared to control over time (-3 wk to 1 wk relative to calving), and this increase in *SLC2A2* expression was paralled with higher lactose yield. Breed, time scale and dietary treatments may explain differences between these studies (Hammon et al., 2009; Goselink et al., 2013; Mach et al., 2013; II), although the role of *SLC2A2* in the liver glucose release in dairy cows is not fully elucidated.

4.3.2 Liver fatty acid metabolism

Liver lipid metabolism is considered a pivotal area in the biology of dairy cow during the transition period. All dairy cows undergo a period of NEB during the early lactation period (Bell and Bauman, 1997). This is compensated by a rise of blood NEFA as a consequence of AT mobilization (Drackley et al., 2001). Plasma NEFA can be utilized as a source of energy by other tissues (Drackley, 1999). Nevertheless, the most critical site for NEFA removal from circulation is the liver (Bell, 1979). NEFA can be converted to acyl-CoA by the action of acyl-CoA synthetase enzyme, then acyl-CoA is transported into mitochondria by carnitine acyl transferase, where acyl-CoA is oxidized into acetyl-CoA via β-oxidation and further oxidized in the citric acid cycle (TCA) to provide energy (Zammit, 1984). Carnitine is an essential cofactor for carnitine palmitoyltransferase-1A (CPT1A) (Drackley et al., 1991). If the amount of acetyl-CoA exceeds the processing capacity of TCA cycle, acetyl-CoA will be used for ketone bodies production (Drackley, 1999). NEFA can be esterified in the liver and exported as TAG within VLDL to extra-hepatic tissues such as the mammary gland (Smith et al., 1997). If the rate of NEFA esterification exceeds the rate of TAG secretion from the liver, lipids start to accumulate (Drackley, 1999). During the postpartum period, the ability of the liver to completely utilize NEFA and secrete TAG within VLDL decreases as the duration and severity of NEB increases (Grummer et al., 2004).

Restricted energy intake or DMI during the dry period may increase DMI of cows postpartum and decrease AT mobilization with lower blood NEFA concentrations (Dann et al., 2006; Douglas et al., 2006; Loor et al., 2006). Overfed cows might have a lower hepatic capacity to completely oxidize NEFA (Loor et al., 2006; Litherland et al., 2011),

and a higher esterification rate of long chain fatty acids with a consequent rise in the liver lipid accumulation (Loor et al., 2006). In the experiment I, *ad libitum* feeding of energy during the far-off period combined with gradually decreasing energy allowance by parturition down-regulated *CPT1A* expression. This may be due to higher plasma insulin concentration early postpartum and lower lipolytic rate prepartum in the overfed group, which in turn would dampen fatty acid β-oxidation (I). Loor et al. (2006) reported that the expression of *CPT1A* decreased and that of diacylglycerol O-acyltransferase 1 (*DGAT1*) increased in cows fed high energy diet compared to cows fed restricted energy during the entire dry period. Andersen et al. (2002) reported lower hepatic fatty acid oxidation during hyperinsulinemic-euglycemic clamp, probably due to the inhibitory effect of insulin on *CPT1A* and the decrease in NEFA mobilization. Furthermore, Rukkwamsuk et al. (1998) and Litherland et al. (2011) reported that overfed cows may have less adapted livers to completely oxidize NEFA around parturition as a consequence of a lower basal lipolytic rate before parturition compared to cows with restricted energy feeding.

Carnitine plays a role in the transport of long chain fatty acids (acyl groups) from cytoplasm to mitochondria for fatty acid oxidation (McGarry and Brown, 1997). SLC22A5 is the most important transporter of carnitine from blood into tissues (Lahjouji et al., 2001). In rodents and pigs, activation of PPARA either by energy deprivation or synthetic agonists led to an increase in hepatic carnitine biosynthesis and uptake (Luci et al., 2006; Maeda et al., 2008). In dairy cows, the transition from pregnancy to lactation was associated with up-regulation of genes involved in carnitine synthesis and uptake in the liver of dairy cows at week 1 postpartum (Schlegel et al., 2012). In contrast, prepartal energy level or the transition from pregnancy to lactation did not affect the mRNA expression of *SLC22A5* in the experiment II, suggesting that the hepatic carnitine uptake from blood stream was not affected by prepartal energy level or lactation.

The periparturient increases in the liver TAG and plasma NEFA concentration were not followed by an increase in hepatic *CPT1A* gene expression (Khan et al., 2014; I; II). This suggests that the influx of NEFA into the liver, a consequence of AT mobilization, was largely directed toward TAG synthesis, and away from fatty acid β-oxidation (Litherland et al., 2011), which was supported by up-regulation of *DGAT1* early postpartum (II). Furthermore, the lack of differences in the liver TAG contents between the groups and down-regulation of *CPT1A* in HIGH (I) suggests that the translocation of long chain fatty

acids into mitochondria by *CPT1A* may not be a key factor influencing the liver TAG. In agreement, Khan et al. (2014) did not observe any time-related changes in the expression of *CPT1A* during the transition period even though there was an increase in the liver TAG and lipid contents.

4.3.3 Liver insulin and growth hormone signalling

Insulin signal transduction is a complex biochemical intracellular process and is composed of three steps. The first step is phosphorylation of a number of proteins including insulin receptors, then activation of secondary messenger intracellular system occurs and the third step is translocation of glucose transporters. Insulin signal transduction is influenced by feeding state, stress, and hormones. Hyperinsulinemia results in downregulation of insulin receptors via increased degradation rates of insulin and its receptors (Hayirli, 2006). Insulin receptor substrate 1 and 2 are expressed in most tissues including the liver, and are known to be responsible for activation of a variety of signal transducing proteins that play important roles in glucose and lipid homeostasis (Saltiel and Kahn, 2001). In the experiments I and II, there were no any treatment- or time-related changes in the mRNA expression of *IRS1* pre- and postpartum between the groups. This might suggest that hepatic expression of *IRS1* has a minor role in the liver insulin sensitivity dynamics in dairy cows around parturition. In periparturient cow, Zachut et al. (2013) observed that insulin function in the liver might be regulated by the amount of *INSR* during the periparturient period.

Growth hormone/insulin like growth factor-1 axis (GH/IGF-1) plays a central role in hepatic metabolic adaptations during the transition period, particularly in carbohydrate and lipid metabolism. It has been shown that plasma IGF-1 was closely related to the insulin status of cow (Butler et al., 2003). During the transition period, the uncoupling of GH/IGF-1 axis mediates the endocrine regulation for nutrient partitioning to fetus and milk synthesis (Bauman, 2000). *IGF-1* was observed to have lower expression after parturition in comparison to the expression before parturition (van Dorland, 2009; Khan et al., 2014), potentially due to lower blood insulin concentration (Butler et al., 2003). In the experiment II, there was no treatment effect on the mRNA expression of *IGF-1* around parturition. In contrast, Khan et al. (2014) observed that overfeeding of energy prepartum (150% of energy requirement for pregnant dairy cows) resulted in higher expression of *IGF-1* before parturition (d -14), but there were no differences postpartum.

The difference of results between the two studies (Khan et al., 2014; II) may be due to different insulin levels, since the ratio of blood insulin between CON and overfed cows was 1:5 in the study by Khan et al. (2014), while in the experiment II; it was 1:1.6. The markedly higher blood insulin prepartum in overfed cows than in control cows in the study by Khan et al. (2014) may be attributed to the relatively high starch intake prepartum. The expression of *IGF-1* was not changed by the transition from pregnancy to lactation in the experiment II. These findings were in contrast to van Dorland et al. (2009), Gross et al. (2011) and Khan et al. (2014). The decrease in blood insulin concentration from prepartum to postpartum was greater in these studies than in the experiment II. In addition to differences in dietary treatments, variation among breeds and the degree of NEB may have contributed to the differences in *IGF-1* expression between these studies (van Dorland et al., 2009; Gross et al., 2011; Khan et al., 2014; II).

4.3.4 Liver inflammation and metabolic transcription regulators

During the transition from late pregnancy to early lactation, dairy cows are in a pro-inflammatory state due to NEB (Trevisi et al., 2012). Excessive adipose tissue deposition and elevated blood NEFA are important risk factors for pro-inflammatory conditions in transition dairy cows (Goff, 2006). It has been demonstrated that there is a causal link between hepatic TAG accumulation and the liver functions and inflammation in dairy cows (Loor et al., 2005). However, the underlying mechanistic link between NEFA and the inflammatory responses in dairy cows during the periparturient period is not fully elucidated. In non-ruminants, interleukin 6 (IL6) is considered as a biomarker for the liver inflammation and diseases (Moshage, 1997). Studies in dairy cows have shown that *IL6* plays a role in the impairment of normal liver function in cases of inflammation and ketosis (Loor et al., 2007; Trevisi et al., 2012). In the experiment II, the mRNA abundance of *IL6* was not different between the treatment groups. In line with this, there was no difference in liver TAG or plasma NEFA between overfed and TMR control cows (II). This may indicate that the liver inflammatory condition was not different between the treatments.

Nuclear factor of kappa polypeptide gene enhancer in B-cells 1 (*NFKB1*) is a key transcriptional regulator in the liver which plays a central role in the regulation of inflammatory and immune responses, and cell survival in humans (Lindström and Bennett, 2005). In the experiments I and II, prepartal dietary energy level did not affect

the expression of hepatic *NFKB1* during the transition period, which may be related to the absence of changes in plasma NEFA and liver TAG between treatments. Loor et al. (2006) reported that cows fed *ad libitum* during the dry period had higher expression of *NFKB1* between d 65 and d 14 before parturition followed by markedly lower expression through d 14 postpartum. Recent study by Graugnard et al. (2013) observed up-regulation of *NFKB1* expression in the liver of cows receiving intramammary LPS challenge, which indicated the acute response to inflammation. In the experiment I, there was no effect of time on *NFKB1* gene expression during the transition period; however, in the experiment II, *NFKB1* mRNA was down-regulated very near parturition. In contrast, Graugnard et al. (2013) reported that the expression of *NFKB1* increased dramatically between -14 and 7 d relative to calving and then decreased at 14 and 30 d after calving. Compared to the experiment II, liver TAG and plasma NEFA were greater in Graugnard et al. (2013) and the experiment I, respectively. This may explain the differences in the expression pattern of *NFKB1* mRNA around parturition between these studies (Graugnard et al., 2013; I; II).

Several studies reported that peroxisome proliferator-activated receptor alpha (*PPARA)* is one of the crucial hepatic nuclear receptors which controls lipid metabolism in the liver (e.g. Mandard et al., 2004; Bionaz et al., 2013). The most important metabolic functions regulated by *PPARA* in the liver are fatty acid oxidation and ketogenesis (Mandard et al., 2004). The expression of *PPARA* and its target genes have been reported to be increased from pregnancy to lactation (Loor et al., 2005; Schlegel et al., 2012). However, other studies have reported up-regulation of *PPARA* target genes without any changes in *PPARA* itself (Carriquiry et al., 2009; van Dorland et al., 2009). Peroxisome proliferator-activated receptor gamma (*PPARG*) is a transcription factor which plays a key role in AT lipogenesis and to lesser extent in the liver (Bionaz et al., 2013). Hepatic *PPARG* was expressed at elevated levels in murine models of obesity or diabetes (e.g. Memon et al., 2000). In mice, *PPARG* plays an important role in the development of fatty liver, and may modulate the amount of liver TAG by regulating the expression of lipogenic genes (Matsusue et al., 2003).

In the experiment II, the expressions of *PPARA* and *PPARG* were not affected by prepartal dietary energy level. It has been observed that minor differences in blood NEFA concentration (van Dorland et al., 2009) or even severe NEB (McCarthy et al., 2010) did not affect hepatic mRNA expression of *PPARG*. Khan et al. (2014) found that the

expression of *PPARA* between d -14 to d 30 relative to calving was not different between overfed and control cows. It has been reported that the large increase in blood NEFA concentration during the transition from pregnancy to lactation was associated with up-regulation of *PPARA* (Loor et al., 2005; Schlegel et al., 2012); however, this response was not found in other studies (Carriquiry et al., 2009; van Dorland et al., 2009). The down-regulation of *PPARA* early postpartum was in line with the declining trend of plasma NEFA at this time (II), and could explain the lack of periparturient increase in *PPARA* target genes (*CPT1A*, *SLC22A5* and *PC*). The observed down-regulation of *PPARG* early postpartum in the experiment II was in agreement with Saremi et al. (2014). It has been demonstrated that the ruminant liver is not a lipogenic tissue (Ballard et al., 1972) and the expression of *PPARG* is relatively low in the liver (Bionaz et al., 2013). Nevertheless, Saremi et al. (2014) suggested that reduced hepatic expression of *PPARG* in dairy cows early postpartum might be linked to enhanced gluconeogenesis or reduced accumulation of lipids in the liver.

Retinol binding protein 4 (RBP4) has multiple roles, e.g. it is an adipokine (Yang et al. 2005) and it has a function as the main transport system for vitamin A (retinol) in plasma (Sivaprasadarao and Findlay, 1988). *RBP4* gene is expressed at least in eight bovine tissues (Merkin et al. 2012; Liao et al. 2014). Plasma retinol decreases sharply before parturition and starts to increase during the first week of lactation (Rezamand et al., 2007), probably due to its accumulation in colostrum rather than milk (Abd Eldaim et al., 2010). The same pattern was also observed for plasma RBP4 (Lindberg et al., 1999), possibly indicating that RBP4 is transferred from cows to their calves through colostrum (Lindberg et al., 1999; Abd Eldaim et al., 2010). Alternatively, lower plasma RBP4 may be due to the suppression of hepatic RBP4 synthesis during the mild inflammation occurred by the process of parturition (Abd Eldaim et al., 2010), although several factors may affect RBP4 synthesis in the liver. It has been shown that plasma RBP4 was reduced with metabolic disorders (Gröhn and Lindberg, 1985), lower prepartal protein intake (Lindberg et al., 1999), intramammary infection (Rezamand et al., 2007), and after LPS administration (Abd Eldaim et al., 2010). In the experiments I and II, overfeeding of energy during the dry period did not affect the expression of hepatic *RBP4* during the transition period. Time effect for hepatic *RBP4* expression was not observed in the experiment I. In the experiment II, there was down-regulation in the mRNA expression of *RBP4* postpartum in comparison to prepartum, in line with Rezamand et al. (2012).

Lindberg et al. (1999) observed that higher protein intake before parturition ensured a more stable plasma RBP4 concentration around parturition. Thus, higher dietary protein level and longer close-up feeding with concentrate in the experiment I than in the experiment II may clarify the inconsistencies in the expression patterns of *RBP4* during the transition period.

There was a significant positive correlation between *RBP4* and *PCK1* in the experiment I which may suggest that *RBP4* might have a role in the regulation of *PCK1* in transition dairy cows. Yang et al. (2005) observed that RBP4 can directly induce *PCK1* expression and reduce insulin action to suppress glucose production in mice liver. However, in the experiment II, *RBP4* mRNA was not correlated with *PCK1* mRNA, but positively correlated with *NFKB1*, *IL6* and *PPARG* mRNA expressions. In humans, Xia et al. (2013) demonstrated that addition of RBP4 to hepatocyte cells directly induced TAG accumulation and increased the expression of lipogenic genes and other transcription factors that play important roles in lipid metabolism including *PPARG* coactivator 1β. Therefore, the down-regulation of *RBP4*, *PPARG* and *NFKB1* mRNA in the liver of dairy cows postpartum compared to prepartum (II) may be an adaptive mechanism to reduce fatty infiltration in the liver postpartum. The role of RBP4 in liver of transition dairy cows warrants further research.

4.4 Insulin resistance status in grazing mares

Obesity and overfeeding of NSC are associated with IR and laminitis in equine (Hoffman et al., 2003; Treiber et al 2006; Frank et al., 2010b). Insulin resistant animals have either resting hyperinsulinemia or higher insulin concentration in response to oral or IVGTT (Frank et al. 2010b; Frank, 2011). The metabolic risk factors for pasture-associated laminitis have been documented in ponies (Treiber et al. 2006, Bailey et al. 2007; Carter et al. 2009) and horses of various breeds (Frank et al., 2006; Vick et al., 2007).

In the experiment III, CG mares had higher basal and peak plasma insulin concentration and greater glucose clearance rate than NG mares at the end of the grazing season. In addition, a greater decrease of plasma NEFA during IVGTT was observed in the CG mares than in NG mares after the grazing season. This indicates that the achieved BW and BCS gains on cultivated high-yielding pasture did not induce IR in AT, and CG mares were able to control plasma glucose. A possible explanation for the above mentioned

results is that the increases in AT depots, particularly visceral fat, in CG mares during the grazing season were not large enough to induce metabolic dysregulation. In line with this, Frank et al. (2006) reported that horses from various breeds with BCS range from 7 to 9 had greater glucose and insulin responses to a combined glucose and insulin tolerance test than those with BCS range from 4 to 6. Recently, Suagee et al. (2013) found that feeding a high glycemic diet did not trigger peripheral IR in Thoroughbred horses when BCS was less than 7.5. It is also likely that the increase in carbohydrate intake from grazing on high-yielding pasture was not high enough or the grazing season was too short to reduce insulin sensitivity of CG mares.

It was also remarkable in the experiment III that several mares showed blunted insulin response during IVGTT which may indicate that these mares had reduced glucose tolerance associated with inadequate pancreatic β cell response to glucose (Firshman and Valberg, 2007), and these mares might have been relatively insulin resistant before the grazing season, as indicated by Si estimates. It has been suggested that pancreatic compensation is impaired in the later stages of IR in horses, and hyperinsulinemia may not be noticed (Kronfeld et al., 2006). Taken together, previous health status or nutritional history may be more significant factor affecting insulin sensitivity in non-obese horses than grazing.

5. CONCLUSIONS

1. Overfeeding energy during the dry period down-regulated key genes linked to hepatic gluconeogenesis (*PC*, *PCK1* and *G6PC*) and fatty acid β-oxidation (*CPT1A*), but the extent of these effects varied depending on prepartal dietary composition (e.g. feeding of cereal grain during the close-up period).
2. *Ad libitum* feeding of grass silage during the whole dry period decreased lipogenesis and exacerbated IR in SAT after parturition (down-regulation of *LEP*, *LPL*, *SCD* and *ADIPOR1/R2*). Decreasing silage allowance and the inclusion of concentrate in the diet during the close-up period dampened the effects of overfeeding on IR and lipogenesis related genes. However, the levels of overfeeding energy during the dry period in both dairy cow experiments did not increase plasma NEFA concentration after parturition in comparison to control.
3. Fattening associated with grazing on cultivated high-yielding pasture induced moderate changes in glucose, insulin and NEFA responses during IVGTT but did not compromise the ability of the horses to control plasma glucose concentrations and AT lipolysis. Moreover, body weight and BCS gains resulted from grazing on cultivated high-yielding pasture were not associated with significant changes in the expression of genes related to IR.

IMPLICATIONS

1. The current results suggest that overfeeding energy during the dry period impacts liver and SAT metabolism of transition dairy cows, even if the cows are not noticeably over-conditioned.
2. Controlling energy intake during the dry period may lead to better transition success in dairy cows.
3. Inclusion of bulky lower quality roughage such as wheat straw at a level of 40% of DM in TMR can be considered as one practical option for controlling energy intake during the dry period.
4. The current findings support the view that obesity and severe hyperinsulinemia are required to produce alterations in IR status in equine.
5. Grazing on high quality pasture 24 h a day does not exacerbate IR in Finnhorse mare when they have normal BCS at the beginning of the summer grazing season.

6. FUTURE RESEARCH

There is growing evidence that NEB is associated with decreased immune function in dairy cows. Thus, it would be interesting to evaluate the effects of feeding high energy diet varying in starch content during the dry period (e.g. grass silage vs. corn silage or level of concentrate during the close-up period) on the immune and inflammatory status of transition dairy cows.

Based on human and laboratory animal studies, it was speculated that visceral AT may play a key role in the development of metabolic diseases compared to SAT. Further research is required to determine the differences in metabolism between visceral fat and subcutaneous fat in order to assess their role in metabolic problems in dairy cows and mares.

Current findings suggest that energy metabolism in non-obese mares might be more influenced by seasonality and health history than pasture feeding. Thus, it would be important to study the effects of seasonality (e.g. wintertime feeding vs. pasture feeding) on energy metabolism to deepen our understanding of molecular pathways which are important in the development of EMS.

A genetic background has been proposed for IR and laminitis in equine. Ponies and certain breeds of horses are more commonly affected than other breeds. Therefore, more studies are needed to evaluate how both genetic background (predisposed vs. non-predisposed breeds) and dietary energy intake (e.g. pasture or concentrate high in NSC content) contribute to the development of IR and laminitis.

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